BAG | Journal of Basic & Applied Genetics

Vol XXXV (1): 65-72; Jan.-July 2024

Journal of the Argentine Society of Genetics

REPRODUCTIVE BIOLOGY AND POLLEN-PISTIL COMPATIBILITY RELATIONSHIPS IN AN ARGENTINIAN COLLECTION OF *Stevia rebaudiana* BERTONI

BIOLOGÍA REPRODUCTIVA Y RELACIONES DE COMPATIBILIDAD POLEN-PISTILO EN UNA COLECCIÓN DE *Stevia rebaudiana* BERTONI EN ARGENTINA

Budeguer C.J.[1], Camadro E.L.[2], Erazzú L.E.[3]

[1]Facultad de Agronomía, Zootecnia y Veterinaria, Universidad Nacional de Tucumán (UNT), Argentina.

[2]Unidad Integrada Estación Experimental Agropecuaria (EEA) "Domingo Pasquale", Instituto Nacional de Tecnología Agropecuaria (INTA)- Facultad de Ciencias Agrarias (FCA), Universidad Nacional de Mar del Plata (UNMdP), Argentina. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

[3]EEA Famaillá, INTA, Tucumán, Argentina.

Corresponding author:
Carlos Budeguer
carlos.budeguer@faz.unt.edu.ar

ORCID 0000-0002-4649-956X

Cite this article as:
Budeguer C.J., Camadro E.L., Erazzú L.E. 2024. REPRODUCTIVE BIOLOGY AND POLLEN-PISTIL COMPATIBILITY RELATIONSHIPS IN AN ARGENTINIAN COLLECTION OF *Stevia rebaudiana* BERTONI. BAG. Journal of Basic and Applied Genetics XXXV (1): 65-72.

Received: 02/04/2023
Revised version received: 06/17/2024
Accepted: 06/24/2024
General Editor: Elsa Camadro
DOI: 10.35407/bag.2024.35.01.05
ISSN online version: 1852-6233

Available online at
www.sag.org.ar/jbag

ABSTRACT

Stevia rebaudiana Bertoni (Asteraceae) is a diploid species (2n=2x=22) with sexual and asexual reproduction. The sexual propagules are seeds produced by cross-pollination (allogamy) whereas the asexual propagules are either vegetative shoots or apomictic seeds. Various authors have reported that allogamy in this species is promoted by the expression of a sporophytic self-incompatibility (SSI) system. To introduce the cultivation of *S. rebaudiana* as a production alternative in Tucumán, Argentina, a germplasm collection of this species was established with accessions from four Argentinian provinces in the Famaillá Agropecuarian Experimental Station (EEA Famaillá), National Institute of Agropecuarian Technology (INTA). The reproductive biology of the collection was studied between 2014 and 2021 to develop strategies for breeding and conservation of these genetic resources. Fifty-six genotypes were analyzed, all of them were 2n=2x=22. Pollen viability was high (69.4 to 99.6%) in all the genotypes except in four of them, which exhibited low viability (36.0 to 51.5%) in 2015 and 2017. Forty-eight genotypic combinations were obtained by manual controlled crosses. In 12 of these combinations, one pollen tube was observed in the style zone and, in four of them, one pollen tube was observed in the embryo sac; these observations indicate, respectively, incompatible and compatible pollen-pistil relationships. Normal plump seeds were obtained in all compatible genotypic combinations. The observed incompatibility might be due to the functioning of the sporophytic homomorphic system and/or a cross-incompatibility system. The observed compatibility will allow the planification of controlled crosses within and between accessions of different geographical origins to generate genetically variable progenies for breeding purposes.

Key words: genetic resources, incompatibility, internal hybridization barriers, pollen viability

RESUMEN

Stevia rebaudiana Bertoni (Asteraceae) es una especie diploide (2n=2x=22) con reproducción sexual y asexual. Los propágulos sexuales son semillas producidas por polinización cruzada (alogamia), mientras que los propágulos asexuales son brotes vegetativos y semillas apomícticas. Varios autores han señalado que la alogamia en esta especie se ve favorecida por la expresión de un sistema de autoincompatibilidad esporofítica (SSI). Para introducir el cultivo de *S. rebaudiana* como alternativa productiva en Tucumán, Argentina, se estableció una colección de germoplasma de esta especie proveniente de cuatro provincias de la Argentina en la Estación Experimental Agropecuaria (EEA) Famaillá, Instituto Nacional de Tecnología Agropecuaria (INTA). Se estudió la biología reproductiva de la colección entre 2014 y 2021 para desarrollar estrategias de mejoramiento y conservación de estos recursos genéticos. Se analizaron 56 genotipos, que fueron 2n=2x=22. La viabilidad del polen fue alta (69,4 a 99,6%) excepto en cuatro de ellos que exhibieron baja viabilidad (36,0 a 51,5%) en 2015 y 2017. Se obtuvieron 48 combinaciones genotípicas mediante cruzamientos controlados manuales. En 12 de estas combinaciones, se observó un tubo polínico en la zona estilar y, en cuatro de ellas, un tubo polínico en el saco embrionario; estas observaciones indican, respectivamente, relaciones polen-pistilo incompatibles y compatibles. Se obtuvieron semillas rellenas normales en todas las combinaciones genotípicas compatibles. La incompatibilidad observada podría deberse al funcionamiento del sistema de autoincompatibilidad homomórfica esporofítica, un sistema de incompatibilidad cruzada, o ambos. La compatibilidad observada permitirá la planificación de cruzamientos controlados dentro y entre introducciones de diferentes orígenes geográficos para generar progenies genéticamente variables con fines de mejoramiento genético.

Palabras clave: recursos genéticos, barreras internas a la hibridación, viabilidad de polen, incompatibilidad.

INTRODUCTION

Stevia rebaudiana Bertoni (Asteraceae) is a diploid species (2n=2x=22) (Galiano, 1987; Frederico *et al.*, 1996; Wulff *et al.*, 1996; Oliveira *et al.*, 2004) of commercial interest due to the presence of glycosylated diterpenes steviol glycosides (SG). *S. rebaudiana* is the only species in genus *Stevia* that exhibits an intense and persistent sweet taste (Soejarto *et al.*, 1982) due to a high SG content in the leaves. These molecules are 250-300 times sweeter than sucrose and non-caloric sweeteners (Ceunen and Geuns, 2013), and are considered as a natural alternative to controversial synthetic sweeteners (Hastoy *et al.*, 2019). In nature, this species has two forms of reproduction: sexual and asexual. Sexual propagules (seeds) are produced by cross-pollination (allogamy), whereas asexual propagules develop from the base of the main stem (vegetative shoots) or by apomixis (apomictic seeds). It has been reported that sexual seeds have low viability, that viable seeds (those with embryos) are dark in color and that the non-viable ones are light and embryoless (Monteiro, 1980; Goettemoeller and Ching, 1999; Yadav *et al.*, 2011).

Commercial field stands are established with asexual propagules obtained by either stem cutting or micropropagation (Kryvenki *et al.*, 2008; Autade *et al.*, 2014; Modi and Kumar, 2018). However, in Argentina, the use of sexual seeds by small-scale farmers results in stands of very variable plants, both morphologically and phenologically.

A major difficulty in seed propagation is the low seed germination percentage, which can vary from 9.0 to 83.0%, depending on seed quality and germination conditions (Macchia *et al.*, 2007; Angelini *et al.*, 2018).

The flowers of *S. rebaudina* are very small (about 3 mm in length), with white petals, hermaphrodites, grouped in capitula which are arranged irregular cymes; they have protandry and mainly entomophilous pollination (Monteiro, 1980; Yadav *et al.*, 2011; Gantait *et al.*, 2018). Studies carried out in Italy determined that the most frequent pollinators were Hymenoptera, Diptera, and Lepidoptera (Martini *et al.*, 2016; Benelli *et al.*, 2017).

Various authors have reported that the species has a sporophytic self-incompatibility (SSI) system (Monteiro, 1980; Gantait *et al.*, 2018), whereas others have not specified whether the observed incompatibility is either sporophytic or gametophytic (Galiano, 1987; Frederico *et al.*, 1996; Yadav *et al.*, 2014; Caponio *et al.*, 2016). In the Asteraceae family, SSI has been described as the characteristic system of self-incompatibility (Frankel and Galun, 1977; Hiscock and McInnis, 2003).

Furthermore, there are contrasting proposals regarding the type of reproduction. Monteiro (1980), in a study of the reproductive biology of three groups of 60 plants each, all from Brazil, concluded that *S. rebaudiana* is an obligate apomictic species. However, Caponio *et al.* (2016) ruled out the existence of apomixis by analyzing the reproductive biology over three years of plants from the Argentinian provinces of Misiones (four) and Entre Ríos (two). The genotypes (and the number of them) characterized in each work were different and the contrasting conclusions do not allow for a generalization; on the contrary, they point out to the necessity of studying the reproductive biology of the accessions conserved at each genebank.

The genotypes of *S. rebaudiana* cultivated in Argentina derive from populations of Paraguay introduced into cultivation in the 1980´s without previous breeding processes, and there are no records on how they were collected. The variety *Criolla* derives from natural populations from the Amambay region in the Paraguay highlands. It is an open-pollinated variety which served as a source of raw material for the development of other current varieties that were incorporated into the international market with the world-wide expansion of the crop, more than two decades ago (Liaudat, 2021).

In order to introduce the cultivation of *S. rebaudiana* as a productive alternative in Tucumán, Argentina, we initiated an active genebank with 75 plants of this species collected from farmers' fields in four provinces (Tucumán, Jujuy, Misiones, and Formosa). These plants were established in the experimental field of the Famaillá Agropecuarian Experimental Station (EEA), National Institute of Agropecuarian Technology (INTA) (27º 01' 05" S, 65º 22' 42" W) in 2013. The aim of the present work was to study the reproductive biology of the active genebank collection of *S. rebaudiana* to develop strategies for breeding and conservation of these genetic resources.

MATERIALS AND METHODS

Plant material

Fifty-six plants of each of four accessions from Tucumán (13), Jujuy (14), Misiones (14) and Formosa (15), were randomly provided by the *S. rebaudiana* INTA Famaillá genebank (SRG). To study pollen-stigma/style compatibility relationships, individual flowers were emasculated and hand-pollinated following an incomplete diallel crossing design. The flowering stages were asynchronous, the flowering behavior of the same plants was variable in each year, and the size of the flowers was very small; thus, 56 plants were selected and each of them was identified with one letter according to origin -T (Tucumán), J (Jujuy), M (Misiones) and F (Formosa)- and numbers for each plant (genotype). Thirty of these plants were further selected for carrying out the crossing work in 2017, 2020, and 2021 because they had high pollen viability (69.4 to 99.6%) (Table 1). The plants were maintained in 2 L individual pots

Table 1. Procedence of the 30 plants of *Stevia rebaudiana* from the INTA Famaillá Genebank used in the study of pollen-stigma/style relationships.

Province	No. plants	Genotype Identification
Tucumán	7	T1.2, T2.3, T2.4, T2.6, T1.8, T2.16, T2.18
Jujuy	8	J3.10, J4.3, J4.5, J5.5, J6.1, J6.5, J8.5, J8.12
Misiones	7	M9.8, M9.9, M9.12, M9.18, M10.4, M11.10, M11.11
Formosa	8	F12.1, F12.2, F12.5, F12.6, F12.8, F12.10, F4, F6

outdoors, with a 1:1 mixture (v:v, peat moss:perlite) and a drip irrigation system.

Methods

Chromosome number and ploidy level

Chromosome number and ploidy level were determined by either root tip chromosome counting (20 plants) or chloroplast counting in the occlusive cells of leaf stomata (36 plants). For the former, root tips were pre-treated with 8-hydroxyquinoline 0.002 M for 4 h, fixed in ethanol 96º: glacial acetic acid (3:1, v/v) for 24 h, and transferred to 70% ethanol until use. For microscopic observations, root tips were hydrolysed in 1N HCl for 10 min at 60º C, rinsed with distilled water and stained with 2% acetic hematoxylin on a glass slide, squashed with a glass bar, covered with a cover slip, and observed under an optical microscope at x1000 magnification.

In plants in which direct chromosome counting could not be performed, the number of chloroplasts in the occlusive cells of the stomata was counted to estimate the ploidy level. In this regard, epidermal tissue was removed with tweezers from the abaxial leaf side for microscopic observations, placed in a glass slide on a drop of lugol (1g KI and 1g I in 100 ml of 70% ethanol), and covered with a cover slip. The number of chloroplasts was determined in either one of the two occlusive cells, in at least 10 stomata/genotype. An average of five to eight chloroplasts per occlusive cell were considered to be indicative of diploidy (Ordoñez *et al.*, 2016).

Pollen viability

For estimating pollen viability, pollen samples were taken at bloom from the dehiscent anthers of four flowers per plant and stained with an 0.5% acetocarmine solution (0.5 g carmine, 45 ml of glacial acetic acid, and 55 ml of distilled water) (Marks, 1954). To this end, a small amount of pollen was placed on a drop of the staining on a glass slide, and covered with a cover slip; then, observations were performed under an optical microscope at x125 magnification. Approximately 300 grains per plant were recorded. Fully stained pollen grains with well-defined contours were considered viable, whereas those that were colorless or poorly stained were considered non-viable.

Pollen-stigma/style compatibility relationships

Crosses between individual genotypes to determine pollen-stigma/style compatibility relationships were carried out following an incomplete diallel mating design, using the plants with high pollen viability as male parents. Flowering stems with receptive stigmata were removed from the plant and pollinated under a stereomicroscope to ensure that enough pollen was deposited on the stigmata. Forty-eight hours after pollination, and following Martin´s (1958) technique, pollinated pistils without ovaries were fixed in FAA (9:0.5:0.5, v/v/v, ethanol: glacial acetic acid: 37% formaldehyde) for at least 1 day. Fixed pistils were rinsed with distilled water, softened in 8 N NaOH solution for 2 h, rinsed again with distilled water, stained with a 0.1% aniline blue solution in tribasic potassium phosphate (0.1N PO_4K_3), mounted in a drop of glycerin on a glass slide, squashed with a cover slip and observed under a fluorescent microscope.

In the compatible genotypic combinations, three to four flowers were manually pollinated for seed formation, in order to confirm that fertilization would indeed occurred. On the other hand, to rule out the formation of viable seeds as a result of autogamy, branches with flower buds were isolated in the plants with voile cloth to avoid the arrival of foreign pollen.

RESULTS

Chromosome number and ploidy level

The number of chromosomes determined by root-tip chromosome counting in 20 plants was 2n=2x=22 (Fig. 1A). The number of chloroplast/occlusive cells of the

Table 2. Percentage of pollen viability -in four years- of 56 genotypes of *Stevia rebaudiana* from various geographic procedences.

Year	Genotype Identification (pollen viability %)[a]
2015	T1.1 (98), T1.2 (89,8), T1.5 (94,0), T1.6 (98,6), T1.8 (87,3), T2.2 (97,0), T2.4 (95,6), T2.5 (99,5), T2.10 (92,4), T2.12 (99,2), T2.15 (95,5), T2.16 (97,3), T2.18 (79,4)
	J3.12 (98,2), J3.14 (97,4), J3.15 (83,8), J3.16 (97,7), J3.2 (99,4), J5.5 (98,8), J6.16 (99), J6.20 (96,8), J6.5 (98,7), J7.5 (94,3), J8.12 (69,4), J8.7 (98,4), J8.9 (98,5)
	M9.1 (99,1), M9.4 (88,5), M9.8 (95,6), M9.12 (97,8), M9.18 (98,3), M10.2 (78,5), M10.6 (89,7), M10.8 (36,5), M10.10 (95,5), M11.2 (98,5), M11.6 (98,0), M11.7 (38,4), M11.11 (38,1)
	F12.1 (51,5), F12.2 (97,1), F12.3 (98,5), F12.4 (97,1), F12.5 (98,7), F12.6 (87,8), F12.7 (99,3), F12.8 (99,3), F12.10 (98,4), F12.11 (99,6), F12.12 (98,3), F12.13 (95,5), F12.14 (79,6), F12.15 (92,4)
2017	T1.1 (98), T1.2 (89,8), T1.6 (98,6), T2.12 (99), T2.18 (79,4)
	J3.14 (97,4), J4.3 (98,8), J5.5 (98,8), J6.20 (96,8), J8.12 (59,9)
	M9.18 (98,3), M10.4 (98,9), M11.6 (98), M11.7 (36), M11.11 (48,5)
	F12.1 (56,1), F12.3 (99,7), F12.4 (90,1), F12.7 (99,3), F12.8 (99,3)
2020	T2.4 (99,5), T2.3 (98,2), T2.16 (98,8), T2.18 (89,7), T9 (88,6)
	J4.5 (98), J 5.16 (99,6), J5.5 (99,7), J6.5 (88,2), J8.12 (100)
	M11.11 (98), M11.10 (87,5), M9.17 (99,4), M9.8 (99,6), M11.7 (83,5)
	F12.1 (89,5), F12.5 (98,4), F12.6 (100), F12.7 (99,8), F12.10 (100)
2021	T1.12 (99,5), T2.4 (97,7), T2.6 (98,4), T1.8 (99,2), T2.18 (99,6)
	J3.10 (99,8), J6.5 (99,5), J6.1 (99,8), J8.5 (97,8), J4.3 (99,2)
	M10.4 (98,3), M11.11 (98,7), M9.18 (99,6), M9.9 (99,2), M9.12 (99,6)
	F12.5 (100), F12.2 (99,7), F12.8 (100), F4 (99,8), F6 (99,7)

[a]High (> 80.0%), medium (80.0 to 60.0%), low (< 60.0%).

stoma in 36 of the 56 analyzed plants varied between six and seven and was, therefore, indicative of the diploid level (Fig. 1B). According to Huamán (1995), an average of six to eight chloroplasts/occlusive cell is indicative of the diploid level in potatoes.

Pollen viability

Pollen viability in the genotypes that reached the flowering stage was estimated in 2015, 2017, 2020 and 2021 (Table 2). High pollen viability was observed in all plants studied in 2017, 2020 and 2021, including those that had low viability in 2015 and 2017.

Pollen-stigma/style relationships

A total of 168 genotypic combinations were carried out in three years: 51 in 2017, 69 in 2020, and 48 in 2021. Between two and three stigmata per floret, and three to four florets per genotype were manually pollinated (Table 3).

In 2017 and 2020, only germinated pollen grains -with pollen tubes approximately of equal length than their diameters- were observed on the stigmata surfaces without stigma penetration (Figure 2A, B). Such genotypic combinations were considered incompatible. In stigmata that were self-pollinated, there was no

Table 3. Number of compatible (C) and incompatible (I) genotypic combinations obtained over three years in controlled crosses of *S. rebaudiana.*

Pollen-stigma/style relationship	Year		
	2017	2020	2021
No. of analyzed genotypic combinations	51	69	48
Type			
C	0	0	12
I	51	69	36

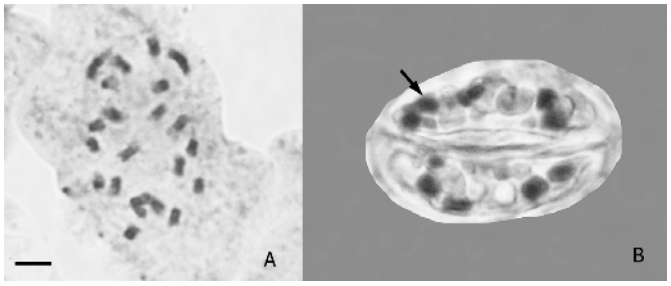

Figure 1. *Stevia rebaudiana.* Determination of the ploidy level by chromosome and chloroplast countings. (A) Mitotic metaphase in plant J4.5 with 2n=2x=22; (B) chloroplasts (arrow) in occlusive cells of a stomata in plant M9.9. Bar = 3 µm.

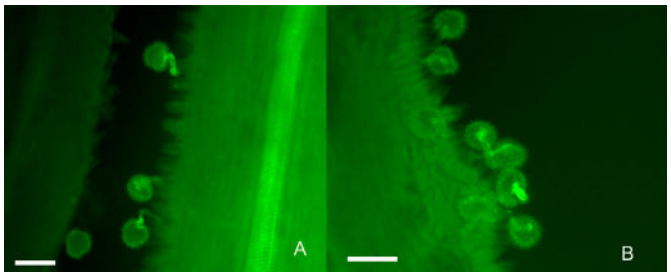

Figure 2. *Stevia rebaudiana.* Incompatibility with pollen tubes arrested in the stigma. (A) genotypic combination M9.18 x T2.18; (B) genotypic combination F12.8 x T2.6. Bar = 20 µm.

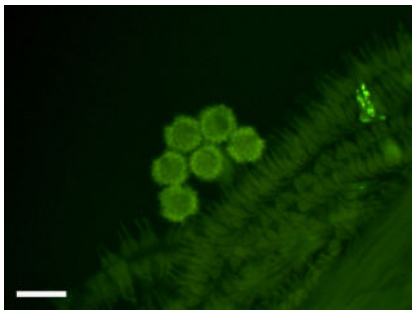

Figure 3. *Stevia rebaudiana.* Self-incompatibility with non-germinated pollen grains on a self-pollinated stigma of plant F12.8. Bar = 20 µm.

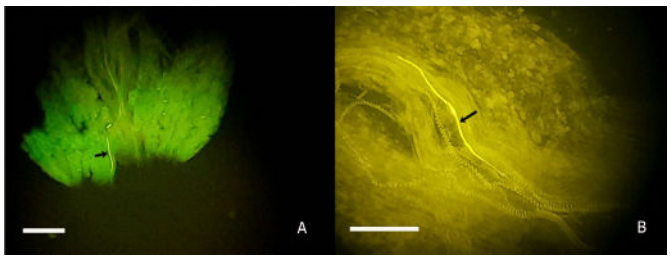

Figure 4. *Stevia rebaudiana.* Pollen tubes (arrow) growing towards the base of the style in compatible genotypic combinations. (A) genotypic combination M10.4 x J3.10; (B) genotypic combination 12.8 x T1.8. Bar = 20 µm.

Figure 5. Seeds of *S. rebaudiana.* (A) clear and empty, obtained from self-fertilization of plant F12.5; (B) dark with embryo, obtained from genotypic combination T2.4 x M9. Bar = 2 mm.

pollen grain germination (Figure 3). On the other hand, one pollen tube was observed in the style in each of 12 genotypic combinations out of the 48 performed in 2021; some of these pollen tubes reached the embryo sac (Figure 4A, B).

From all of the pollen-pistil compatible genotypic combinations, well-formed seeds were obtained (Table 4). All the plants whose florets were isolated with voile cloth to favor self-pollination produced empty, light-colored seeds without embryos (Figure 5A, B).

Table 4. Number (No.) of pollinated flowers and No. of seeds obtained in compatible genotypic combinations of *S. rebaudiana*.

Genotypic combination	No. pollinated flowers	No. seeds
F6 x T2.6	3	2
F12.8 x T1.8	3	3
M10.4 x J3.10	4	3
M9.18 x J6.5	4	2
J3.10 x M10.4	3	1
J6.5 x M9.18	3	3
T2.4 x M9.9	3	2
T2.6 x F12.5	3	2
F4 x F12.5	4	3
M9.18 x F4	4	4
J6.1 x M9.12	4	3

DISCUSSION

The chromosome number determined in root tip cells (2n=2x=22) in all the analyzed plants is the same reported by Frederico *et al.* (1996) for *S. rebaudiana* from Brazil. It is also the same reported by Oliveira *et al.* (2004) for 11 genotypes of *S. rebaudiana* from CENARGEN/EMBRAPA, Brazil. The basic number (x= 11) is common in most South American species of the *Stevia* genus (Galiano, 1987; Frederico *et al.*, 1996). Chloroplast counting in the stomata occluding cells was an effective technique to estimate the diploid level of the remaining analyzed plants; even though its application does not give an exact result, it allows for the distinction among ploidy levels (Poulsen Hornum and Camadro, 2021).

The percentage of pollen viability was high over the four years, with the exception of four plants in 2015 and 2017. Abdullateef *et al.* (2012) reported that pollen viability of three introductions of *S. rebaudiana* grown in the field in Malaysia ranged from 88.6 to 93.3%. Similarly, Caponio *et al.* (2016) reported 94.8 to 97.9% of pollen viability in plants from the provinces of Misiones (four) and Entre Rios (two), Argentina. Likewise, Monteiro (1980), working with 30 plants in Campinas, Brazil, determined that pollen viability ranged from 55% to 65%.

The analysis of pollen viability is of fundamental importance to identify fertile male parents to use as pollinators in breeding. Previously, Budeguer *et al.* (2018) reported abnormalities in meiosis and at tetrad stage in plants M10.8, M11.7, M11.11 and F12.1, which would explain their low pollen viability. In the present study, low pollen viability was observed in very few plants, and only in 2015 and 2017; these results could be attributed to the expression of genotype x environment interactions. In any case, pollen viability would not represent a problem in planning the crossing work with the SGR´s collection conserved at EEA Famaillá.

For the study of the pollen-stigma/style compatibility relationships, the manual pollination technique was extremely difficult to perform due to the very small size of the flowers and the poor adherence of the pollen on the stigmata; thus, a fine-tuning of the technique was previously required to perform the crosses on cut stems under a stereomicroscope. Flowering asynchrony in each year was an additional difficulty in the obtainment of the genotypic combinations.

Despite the difficulties mentioned above, 168 genotypic combinations were achieved in three years (2017, 2020, and 2021). Flower fixation 24 h after hand pollination proved adequate to observe pollen tube growth in the ovary. Caponio *et al.* (2016) studied the germination of pollen grains *in situ* in three ecotypes of *S. rebaudiana*; they observed that 88% of the attached pollen grains developed pollen tubes 8 h after

pollination, and that pollen tubes reached the lower third of the ovary 16 h later.

In 2021, pollen tubes were observed in the base of the style and growing into the ovary in 12 of the 48 genotypic combinations; in the remaining combinations, only tubes above the stigmatic papillae were observed, none of which penetrated the stigmata. This could be due to the functioning of the sporophytic homomorphic self-incompatibility system characteristic of the Asteraceae family (Frankel and Galun, 1977; Allen *et al.*, 2011; Gantait *et al.*, 2018), a cross-incompatibility system, or both. Frankel and Galun (1977) pointed out that the SSI is controlled by one multi-allelic *S*-locus (*S*-haplotype), being the reaction of the n pollen determined by the 2n genotype of the sporophytic tissue in which it was formed; thus, upon self-pollination all pollen grains of a plant will exhibit the same incompatibility reaction regardless of their own genotypes. However, the *S*-alleles may exhibit dominance/independence relationships that may differ in the pollen and pistils of the same plant, generating complex incompatibility reactions (Frankel and Galun, 1977; Hiscock and McInnis, 2003). Pollen-pistil cross-incompatibility -as observed in other species of various families of Dicots and Monocots (see examples in Marcellán and Camadro, 1996; Arias *et al.*, 2003; Ibañez and Camadro, 2014; Maune *et al.* 2018)- could also explain the observed incompatible reactions. Nevertheless, the simultaneous action of both systems cannot be discarded.

The results of this work allow us to conclude that the collection conserved in the SRG at EEA Famaillá, Tucumán, are diploid (2n=2x=22) and also allogamous because no seeds were formed in the flowers isolated with voile cloth. This lack of seed formation does not allow speculation on the possible apomictic behavior reported by Monteiro (1980) for the species. Pseudogamous apomixis requires the growth of the pollen tube in the embryo sac and the discharge of the generative cells into the central cell for the formation of the first endospermic cell after fusion of their respective nuclei, a phenomenon that cannot take place if a sporophytic incompatibility system with the same type of *S*-allele expression in the maternal and paternal tissues is acting.

In the compatible genotypic combinations, it was possible to achieve fertilization and viable seed formation, so it would be possible to plan crosses between genotypes from the SRG, taking into consideration different geographical origins, to increase the chances of obtaining genetically variable progenies. It is also advisable to previously determine the viability of the pollen of the plants that would act as paternal progenitors.

REFERENCES

Abdullateef R.A., Zakaria N.H., Hasali N.H., Osman M. (2012) Studies on pollen viability and germinability in accessions of *Stevia rebaudiana* Bertoni. Int. J. Biol. 4(3): 72-79.

Allen A.M., Thorogood C.J., Hegarty M.J., Lexer C., Hiscock S.J. (2011) Pollen–pistil interactions and self-incompatibility in the Asteraceae: New insights from studies of *Senecio squalidus* (Oxford ragwort). Ann. Bot. 108(4): 687–698.

Angelini L.G., Martini A., Passera B., Tavarini S. (2018) Cultivation of *Stevia rebaudiana* Bertoni and Associated Challenges. In Mérillon J.M. & Ramawat K.G. (Eds.) Sweeteners. Springer International Publishing, pp. 35–85. https://doi.org/10.1007/978-3-319-27027-2_8

Arias M., Camadro E.L., Díaz Ricci J., Castagnaro A.P. (2003) Breeding barriers between the cultivated strawberry, *Fragaria* x *ananassa*, and related wild germoplasm Euphytica, 136: 139-149.

Autade R., Fargade S., Borhade P., Udmale S., Choudhary R. (2014) In Vitro Propagation of *Stevia rebaudiana* (Bert.) a Natural, Non-Caloric Sweetener Herb. J. Cell Tissue Research, 14(3): 4659–4664.

Benelli G., Canale A., Romano D., Flamini G., Tavarini S., Martini A., Angelini L. G. (2017) Flower scent bouquet variation and bee pollinator visits in Stevia rebaudiana Bertoni (Asteraceae), a source of natural sweeteners. Arthropod-Plant Inte. 11(3), 381–388.

Budeguer C.J, Camadro E.L, Erazzú L.E. (2018) Pollen viability and meiotic irregularities in a collection of *Stevia rebaudiana* (Bertoni) Bertoni cultivated in Tucumán, Argentina. BAG. Journal of Basic and Applied Genetics, 29(2): 49-57.

Caponio I., Torres S.C., Andersson G., Norrmann G.A. (2016) Aspectos de la biología reproductiva de *Stevia rebaudiana* (Asteraceae). Bonplandia, 25(1): 59. https://doi.org/10.30972/bon.2511279.

Ceunen, S., Geuns, J.M.C. (2013) Steviol Glycosides: Chemical Diversity, Metabolism, and Function. J. Nat. Prod. 76: 1201–1228.

Frankel R., Galun E. (1977) Pollination Mechanisms, Reproduction and Plant Breeding (Vol. 2). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-81059-6.

Frederico A.P., Ruas P.M., Marin-Morales M.A., Ruas C.F., Nakajima, J.N. (1996) Chromosome studies in some *Stevia*. Cav. (Compositae) species from Southern Brazil. Braz. J. Genet. 19: 605–609.

Galiano N. (1987) Estudios cromosómicos en especies argentinas de *Stevia* (Compositae). Darwiniana, 28: 311–315.

Gantait S., Das A., Banerjee J. (2018) Geographical distribution, botanical description and self-incompatibility mechanism of genus *Stevia*. Sugar Tech. 20: 1–10.

Goettemoeller J., Ching A. (1999) Seed germination in *Stevia rebaudiana.* In: Janick J. (Ed.) Perspectives on new crops and new uses. ASHS Press, Alexandria, VA, pp. 510–511.

Hastoy, C., Cosson, P., Cavaignac, S., Boutie, P., Waffo-Teguo, P., Rolin, D., Schurdi-Levraud, V. (2019) Deciphering performances of fifteen genotypes of *Stevia rebaudiana* in southwestern France through dry biomass and steviol glycoside evaluation. Ind. Crops Prod. 128: 607-619.

Hiscock S.J., McInnis S.M. (2003) The Diversity of Self-Incompatibility Systems in Flowering Plants. Plant Biol. 5(1): 23–32. https://doi.org/10.1055/s-2003-37981.

Huamán Z. (1995) Técnicas citológicas para determinar el número cromosómico y la fertilidad de las papas. Guía de Investigación CIP 10. Centro Internacional de la Papa, Lima, Perú.

Ibañez M.S., Camadro E.L. (2014) Reproductive behavior of the wild carrots *Daucus pusillus* and *D. montanus* from Argentina Botany, 93: 279-286. dx.doi.org/10.1139/cjb-2014-0243.

Kryvenki M., Kosky R.G., Guerrero D., Dominguez M., Reyes M. (2008) Obtención de callos con estructuras embriogénicas de *Stevia rebaudiana* Bert. en medios de cultivo semisólidos. Biotecnol. Veg. 8(2): 91–98.

Liaudat S. (2021) *Stevia*. Conocimiento, propiedad intelectual y acumulación de capital . Prometeo Libros. 336 p.

Macchia M., Andolfi L., Ceccarini L., Angelini L.G. (2007) Effects of Temperature, Light and Pre-Chilling on Seed Germination of *Stevia rebaudiana* (Bertoni) Bertoni Accessions. Ital. J. Agron. 2(1): 55. https://doi.org/10.4081/ija.2007.55.

Marcellán O.N., Camadro E.L. (1996) Self- and cross- incompatibility in *Asparagus officinalis* and *A. densiflorus* cv. Sprengeri. Can. J. Bot. 74 (10): 1621-1625.

Marks G.E. (1954) An aceto-carmine glycerol jelly for use in pollen fertility counts. Stain Technol. 29: 277. https://doi.org/10.3109/10520295409115483.

Martin F.W. (1958) Staining and Observing Pollen Tubes in the Style by Means of Fluorescence. Stain Tech. 34(3): 125–128. https://doi.org/10.3109/10520295909114663.

Martini A., Tavarini S., Macchia M., Benelli G., Canale A., Romano D., Angelini L.G. (2016) Influence of insect pollinators and harvesting time on the quality of *Stevia rebaudiana* (Bert.) Bertoni seeds. Plant Biosyst. 151(2): 341–351. https://doi.org/10.1080/11263504.2016.1174173.

Maune J.F., Camadro E.L., Erazzú L.E. (2018) Cross-incompatibility and self-incompatibility: unrelated phenomena in wild and cultivated potatoes? Botany, 96 (1): 33 - 45.

Modi A., Kumar N. (2018) Conventional and Biotechnological Approaches to Enhance Steviol Glycosides (SGs) in *Stevia rebaudiana* Bertoni. In: Kumar N. (Ed.) Biotechnological Approaches for Medicinal and Aromatic Plants. Springer Singapore, pp. 53–62. https://doi.org/10.1007/978-981-13-0535-1_3.

Monteiro R. (1980) Taxonomia e Biologia da Reproduçao de *Stevia reabaudiana* (Bert.) Bert. Tesis Doctoral. Universidad de Campinas, São Paulo, Brasil.

Oliveira V.M., Forni-Martins E.R., Magalhães P.M., Alves M.N. (2004) Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). Genet. Mol. Biol. 27(2): 215–222.

Ordoñez, B., Orrillo, M., Bonierbale, M. (2016) Manual biología reproductiva y citológica de la papa. Lima (Perú). Centro Internacional de la Papa (CIP). ISBN 978-92-9060-477-8. 2a ed. 65 p.

Poulsen Hornum A., Camadro E.L. (2021) Expression of internal reproductive barriers in a germplasm bank accession of the wild potato *Solanum chacoense* Bitter in three ex situ regeneration cycles. Genet. Resour. Crop Evol. 68(3): 915–938. https://doi.org/10.1007/s10722-020-01034-y.

Soejarto D.D., Kinghorn A.D., Farnsworth N.R. (1982) Organoleptic evaluation of Stevia leaf herbarium samples for sweetness. J. Nat. Prod. 45: 590–599.

Wulff A.F., Hunziker J.H., Escobar A. (1996) Estudios cariológicos en Compositae VII. Darwiniana, 34: 213–231.

Yadav A.K., Singh S., Dhyani D., Ahuja P.S. (2011) A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. Can. J. Plant Sci. 91(1): 1–27.

Yadav A.K., Singh S., Rajeev R. (2014) Self-incompatibility evidenced through scanning electron microscopy and pollination behaviour in *Stevia rebaudiana*. Indian J. Agric. Sci. 84(1), 93–100.

FUNDING

This study was partially funded by Instituto Nacional de Tecnología Agropecuaria (INTA; 2023-PD-L01-I071) and Universidad Nacional de Tucumán (PIUNT A705).